Levy, A. L. (1955), *Methods Biochem. Anal. 2*, 360.
MacDonnell, L. R., Silva, R. B., and Feeney, R. E. (1951), *Arch. Biochem. Biophys. 32*, 288.
Mills, G. L. (1952), *Biochem. J. 50*, 707.
Niu, C.-I., and Fraenkel-Conrat, H. (1955), *J. Am. Chem. Soc. 77*, 5882.
Sanger, F. (1945), *Biochem. J. 39*, 507.
Sjoquist, J. (1953), *Acta Chem. Scand. 7*, 447.
Spero, L., Stefanye, D., Brecher, P. I., Jacoby, H. M., Dalidowicz, J. E., and Schantz, E. J. (1965), *Biochemistry 4*, 1024.
Tsugita, A., Gish, D. T., Young, J., Fraenkel-Conrat, H., Knight, C. A., and Stanley, W. M. (1960), *Proc. Natl. Acad. Sci. U. S. 46*, 1463.

Ascaris Cuticle Collagen: on the Disulfide Cross-Linkages and the Molecular Properties of the Subunits*

O. Wesley McBride† and William F. Harrington

ABSTRACT: The subunit polypeptide chains of neutral salt-soluble *Ascaris* cuticle collagen (mol wt 900,000) are covalently linked by disulfide cross bridges. Reducing agents produce a rapid transition in the molecular properties of this collagen in the presence or absence of denaturing conditions. (1) The molecular weight decreases by a factor of 10–15. (2) The reduced viscosity (η_{sp}/c) falls to values in the range 0.5–1 dl/g. (3) The sedimentation coefficient ($s^0_{20,w}$) decreases from 7.0 to about 2.2 S. (4) The alkylated product (RCM *Ascaris*) shows a quantitative conversion of cystine to *S*-carboxymethylcysteine. Nevertheless, in the absence of denaturing agents >75% of the collagen-fold conformation is retained by the subunit polypeptide chains when the SS bridges are cleaved as judged by optical rotation studies. Gel filtration and high- and low-speed sedimentation equilibrium studies indicate that RCM *Ascaris* is relatively homogeneous with respect to size with a molecular weight of 62,000. At low temperature the collagen-fold conformation is regenerated in RCM *Ascaris* and the helix–coil transition in this material occurs without any change in molecular weight. Reduction and alkylation of the insoluble collagen matrix of *Ascaris* cuticle disperse virtually all of the collagen into solution, and the reduced alkylated product is indistinguishable in physical and chemical properties from that prepared from the neutral salt-soluble material.

Reoxidation of reduced *Ascaris* collagen gives a heterogeneous mixture of particle sizes, but the optical rotatory properties of the native protein are regenerated. The structure of the native collagen is considered in terms of the physical properties of the subunits.

Neutral salt-soluble collagen isolated from the cuticle of the invertebrate *Ascaris lumbricoides* exhibits a number of unusual physical and chemical properties. The molecular weight of this material, estimated at about 900,000 (Josse and Harrington, 1964), is significantly higher than that of the vertebrate tropocollagens. Moreover, the individual polypeptide chains of the structure appear to be covalently cross-linked since the molecular weight remains unchanged on denaturation, and since all of the physical properties of the native structure are completely regenerated at low temperature following thermal denaturation. Aside from the unusual pyrrolidine composition (29% proline and 2% hydroxyproline), *Ascaris* cuticle collagen exhibits an anomalously low glycine content (28%) and a small, but significant amount of half-cystine. The consistent presence of half-cystine residues in amino acid analyses of purified *Ascaris* collagen has prompted us to investigate the effect of reductive cleavage agents on the properties of this material. As we will demonstrate below, the polypeptide chains of *Ascaris* cuticle collagen are cross-linked through disulfide bridges. This report will focus on the properties of the subunit polypeptide chains derived from *Ascaris* on reductive cleavage of the disulfide bridges and will consider the significance of these findings in terms of the structure of the native collagen. A preliminary

* Publication No. 497 of the McCollum-Pratt Institute, The Johns Hopkins University, Baltimore, Maryland. *Received November 28, 1966.* This work was supported by a U. S. Public Health Service research grant (AM 04349) from the National Institute of Arthritis and Metabolic Diseases.

† Present address: National Cancer Institute, National Institutes of Health, Bethesda, Md. Work was carried out during the tenure of a U. S. Public Health Service special postdoctoral fellowship.

account of our findings has already been published (McBride and Harrington, 1965).

Materials and Methods

Preparation and Purification of Ascaris Collagen. A. lumbricoides was obtained from hog intestines. All preparative procedures were carried out at 5°. The basis of purification was the achievement of the highest possible ratios of hydroxyproline to protein and proline to protein.

PURIFICATION OF "NATIVE" SOLUBLE *Ascaris* COLLAGEN. The procedure used was basically that of Josse and Harrington (1964). The washed, minced cuticles were ground to a slurry in a mortar and pestle and extracted with 0.5 M NaCl to remove the bulk of the soluble noncollagenous proteins. The residue was suspended in 0.5 M NaCl and homogenized in a motor-driven, ground-glass homogenizer. The pooled homogenates were stirred for 24–48 hr. After centrifugation at 15,000*g* for 20 min, the clear supernatant liquid was collected and the large, white opaque gel (A) was saved. The supernatant containing the soluble *Ascaris* collagen was further purified by two successive $(NH_4)_2SO_4$ precipitations. It was usually necessary to add $(NH_4)_2SO_4$ to a final concentration of 25% (occasionally 30% depending upon the protein concentration) in order to precipitate most of the collagen. The ratio of proline and hydroxyproline to protein reached constant levels after two precipitations and very little protein remained in solution on any subsequent $(NH_4)_2SO_4$ treatment. The final pellet was redissolved in 0.2 M NaCl with stirring for 48 hr, and dialyzed against repeated changes of 0.2 M NaCl over several days. This material was centrifuged at 70,000*g* for 1 hr in a no. 30 rotor of a Spinco Model L resulting in a clear, very viscous supernatant (soluble native collagen at concentrations of 1.0–2.5 mg/ml) containing 50–75% of the protein and a clear, colorless, gel precipitate (B). Neither of the gels (A or B) could be dissolved by alteration of the pH or by addition of denaturing agents such as 5 M guanidine·HCl. If the gel A were repeatedly rehomogenized and the purification procedure were repeated, a very small and diminishing increment of soluble collagen was obtained.

PREPARATION OF REDUCED CARBOXYMETHYLATED (RCM) *Ascaris* COLLAGEN. A solution of native *Ascaris* collagen (about 0.1%) in 5 M guanidine·HCl, 0.4 M Tris·HCl, and 0.2% Na-EDTA (final apparent pH 8.6) was flushed with a stream of N_2 and β-mercaptoethanol was added to make a final concentration of 1%. The vessel was stoppered and reduction was permitted to proceed for 6–24 hr at 25°. The flask was again flushed with a stream of N_2 and a fresh solution of 10 ml of iodoacetic acid in 1 N NaOH (2.68 g/10 ml) was added/1 ml of mercaptoethanol (nearly stoichiometric amount) in the reaction mixture (Crestfield *et al.*, 1963). After the alkylation continued for 30 min in the dark, a twofold excess of mercaptoethanol was added to react with any remaining iodoacetate and the solution was dialyzed extensively *vs.* 0.2 M NaCl. Gels A and B were reduced and alkylated in a similar manner. All solutions were centrifuged at 70,000*g* for 1 hr and the clear, somewhat viscous, supernatant solutions were used for further study.

Chemicals. All chemicals were reagent grade. Guanidine·HCl was purified by recrystallization from methanol following treatment with activated charcoal. Anthrone and *p*-dimethylaminobenzaldehyde were recrystallized from benzene and light petroleum ether (bp 30–60°), respectively. *p*-Mercuribenzoate was purified by repeated precipitation with dilute HCl and resuspension in dilute NaOH (Boyer, 1954). CM-50 Sephadex (Pharmacia) and CM-cellulose (Whatman) were washed with alkali, acid, and distilled water before used. Water was deionized and glass distilled.

Protein Concentrations. The microbiuret procedure of Zamenhof (1957) was used routinely for determination of protein concentrations. This procedure was standardized for *Ascaris* collagen solutions by micro-Kjeldahl analyses using the percentage nitrogen values calculated from the amino acid composition. The microbiuret assay could not be performed in the presence of guanidine, urea, LiBr, Tris, or β-mercaptoethanol. Citrate solutions give slightly high blank values. Most other salts do not interfere. Protein solutions in 5 M guanidine·HCl were prepared (using density data on aqueous guanidine·HCl solutions (Kielley and Harrington, 1960)) by adding a weighed amount of the solid salt to a collagen solution of known concentration. Solutions which included other interfering reagents were prepared by adding appropriate weights or volumes of the reagents to weighed amounts of collagen solutions of known concentration followed by dilution to a known final volume.

Chemical Methods. Sulfhydryl groups were measured by the method of Ellman (1959) involving the reaction with 5,5′-dithiobis(2-nitrobenzoic acid). Measurements were also made by titration with *p*-mercuribenzoate as described by Boyer (1954). Carbohydrate was determined by the anthrone method of Loewus (1952) with D-glucose as a standard.

Samples of lyophilized, deionized protein were prepared for amino acid analysis by hydrolysis in 6 N HCl in evacuated, sealed tubes at 110° for 24 hr. The amino acid composition was determined according to the technique of Spackman *et al.* (1958). Analyses were performed at 55° using a standard 6-cm column (accelerated beaded resin) for separation of the basic amino acids and a 50-cm column for the neutral and acidic amino acids. The results were corrected for amino acid destruction during hydrolysis using the values of Piez *et al.* (1960). Separation of the neutral and acidic amino acids was also carried out on the 50-cm column using a programmed 30–50° run to separate hydroxyproline. Better resolution of the other amino acids was obtained using the routine procedure at 55°. The 30–50° long-column analyses were not routinely performed on hydrolysates of RCM *Ascaris* collagen, since it was found that hydroxy-

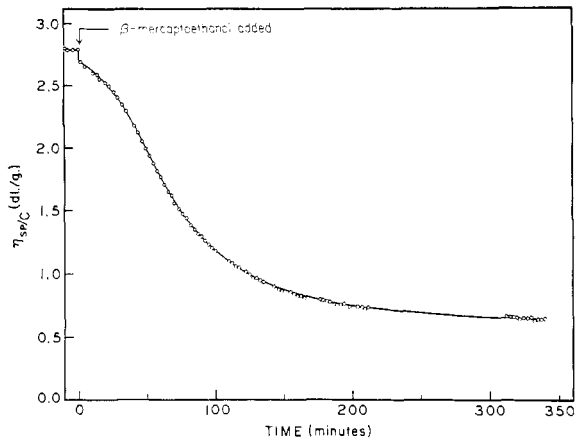

FIGURE 1: Reduced viscosity of *Ascaris* collagen in 5 M guanidine·HCl (25°) following addition of β-mercaptoethanol (0.5%) at pH 4.6. Solvent also included NaCl (0.12 M).

proline and SCMC[1] would not be adequately separated by this procedure. Since there was almost twice as much SCMC as hydroxyproline present in these hydrolysates, and in view of the fact that the color yield for hydroxyproline is only about 4% of that for SCMC at 570 mμ, the presence of hydroxyproline did not interfere with the accurate determination of SCMC. During the preparative procedure, proline and hydroxyproline determinations were performed on neutralized acid hydrolysates by the method of Chinard (1952) and Woessner (1961), respectively.

Viscosity measurements were made in an Ostwald capillary viscometer with an average shear gradient of 300 sec^{-1} and an outflow time of 85 sec for 2 ml of water at 25°. The temperature of the viscometer water bath was maintained within ±0.01° of the reported temperature. Viscosity results are given as reduced viscosity η_{sp}/c, where η_{sp} is the specific viscosity and c is the protein concentration in g/100 ml.

Sedimentation Studies. The Rayleigh interference optical system of the Spinco Model E ultracentrifuge was used for sedimentation equilibrium studies and the schlieren optical system for sedimentation velocity experiments. Velocity sedimentation studies were carried out with rotor speeds of 59,780 rpm. In most experiments with RCM *Ascaris* collagen in 5 M guanidine·HCl, a sharp initial sedimenting boundary was formed by layering solvent over the solution in a synthetic boundary cell. The observed sedimentation coefficients were corrected to the standard conditions of water at 20°. In all sedimentation studies an apparent specific volume of $\bar{V} = 0.71$ cc/g has been assumed for *Ascaris* and RCM *Ascaris* (Josse and Harrington, 1964).

[1] Abbreviations used: SCMC, *S*-carboxymethylcysteine; PMB, *p*-mercuribenzoate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

The molecular weight of RCM *Ascaris* collagen in 0.2 M NaCl was determined from two general types of sedimentation equilibrium experiments.

1. LOW-SPEED SEDIMENTATION EQUILIBRIUM EXPERIMENTS were performed according to the method of Van Holde and Baldwin (1958) employing 1-mm liquid columns layered over FC 43 (perfluorotributylamine, Minnesota Mining and Manufacturing Co.). Rotor velocities of 11,272 rpm were routinely used. Higher rotor velocities than usual were necessary to permit measurement of the apparent molecular weight over as wide a concentration span as possible without increasing the time necessary to achieve equilibrium by increasing the column height. Apparent heterogeneity of particle mass was also accentuated by this procedure. Initial concentrations, c_0 in fringes, of the protein solutions were determined separately by means of a double-sector synthetic boundary cell; two or three runs were made on one solution and c_0 was calculated for subsequent weighed dilutions. The time required for equilibrium (assuming a molecular weight of 63,000 for RCM *Ascaris*) was estimated to be 6–7 hr (Van Holde and Baldwin, 1958). However, experiments were usually performed for 16 hr and it was determined in each case that equilibrium had been attained by demonstrating that the mass distribution within the cell had become invariant with respect to time. The methods for determining molecular weight by sedimentation equilibrium require either a knowledge of the concentration of macromolecules at some point in the cell or the implicit assumption of conservation of mass within the cell. We have chosen to utilize the white light method (Schachman, 1963; Richards and Schachman, 1959) to determine directly the hinge point (the radial coordinate where $c = c_0$). This procedure eliminates any error in concentration introduced by removal of mass from the liquid column. The hinge point was usually located approximately 55% of the radial distance down the liquid column measured from the meniscus to the base. From the equations of Van Holde and Baldwin (1958), the location of the hinge point was estimated 52% of the distance down the liquid column. Log c was plotted against r^2 and nearly all plots showed some upward curvature indicative of mass heterogeneity. The apparent molecular weight over the entire column was calculated by the procedure of Lansing and Kraemer (1935). Apparent weight-average molecular weights were also determined from the slopes of the log c *vs.* r^2 plots at the meniscus, midpoint, and base using the relationship

$$M_{w\,app} = \frac{2RT\mathrm{d}\ln c}{(1 - \bar{v}\rho)\omega^2\mathrm{d}r^2}$$

The z-average molecular weights over the whole column were also computed according to the method of Lansing and Kraemer (1935).

2. HIGH-SPEED SEDIMENTATION EQUILIBRIUM EXPERIMENTS. These experiments were carried out with 3-mm

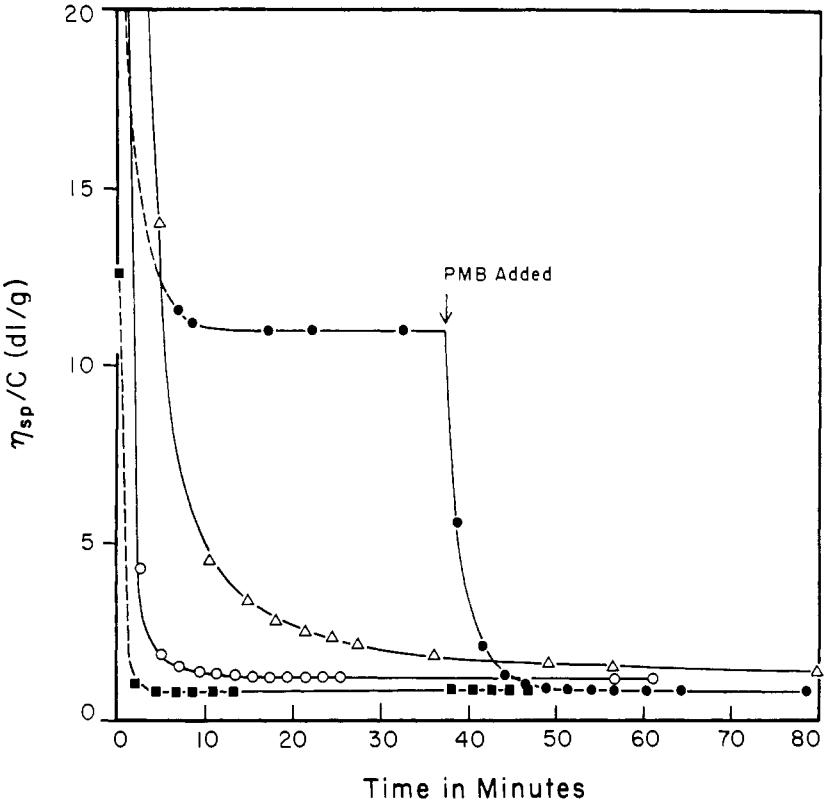

FIGURE 2: Reduced viscosity of *Ascaris* collagen as a function of time in the presence of various reducing agents at 25°. (●) 0.18 M Na_2SO_3, pH 9; PMB = 0.0018 M. (Δ) 0.06 M β-mercaptoethanol, pH 8. (○) 0.05 M dithiothreitol, pH 8. (■) 0.6 M β-mercaptoethanol, pH 10. For details of solvent system refer to Table I.

liquid columns layered over FC-43 using the procedure outlined by Yphantis (1964). A rotor velocity of 24,630 rpm was routinely employed but some experiments were performed at velocities ranging from 17,000 to 30,000 rpm. In experiments where high concentrations of salt were present, considerable care was taken to ensure that the volume of FC-43 and also the volume of solution and solvent in each sector of the cell were the same since significant differences in salt distribution will result from unequal liquid columns at high rotor velocities. Protein concentrations employed were 0.01–0.025%.

Optical Rotation. Most of the measurements were made using a Model 80 Rudolph spectropolarimeter equipped with xenon and mercury light sources and an oscillating polarizer prism. The temperature in jacketed polarimeter tubes in series with a variable temperature bath was usually maintained within ±0.1° and it was estimated by averaging the temperatures of influx and efflux circulant. All rotations were corrected for solvent refractive index (Abbe refractomer, 25°) and reduced to a water environment. Deep ultraviolet measurements and most specific rotations for dispersion plots were made on a Cary Model 60 recording spectropolarimeter utilizing water-jacketed cells. Dispersion data were plotted according to the method of Yang and Doty (1957) and obeyed a one-term Drude equation over the spectral range 260–600 mμ.

Gel Electrophoresis. Analytical electrophoresis was carried out on acrylamide (5%) gel columns according to the procedure of Ornstein (1964) and Davis (1964). Ammonium persulfate was electrophoretically removed from the gels prior to application of the samples.

Results

Effect of Reducing Agents on the Physical Properties of Ascaris Cuticle Collagen. VISCOSITY AND SEDIMENTATION CHANGES IN THE PRESENCE OF DENATURING AGENTS. The effect of β-mercaptoethanol on the viscosity of *Ascaris* collagen in aqueous 5 M guanidine·HCl is presented in Figure 1. In this experiment β-mercaptoethanol (0.01 ml) was added to 2 ml of an unbuffered (pH 4.6) *Ascaris* collagen solution (1.26 mg/ml) in 0.12 M NaCl–5 M guanidine·HCl which had been equilibrated at 25° in a capillary viscometer. Following addition of the β-mercaptoethanol, the reduced viscosity was observed to decrease from 2.8 to approximately 0.57 dl/g with a half-time of the over-all reaction of about 65 min. The time dependence of viscosity change does not follow any simple kinetic order. The rate of change of viscosity increases with time reaching

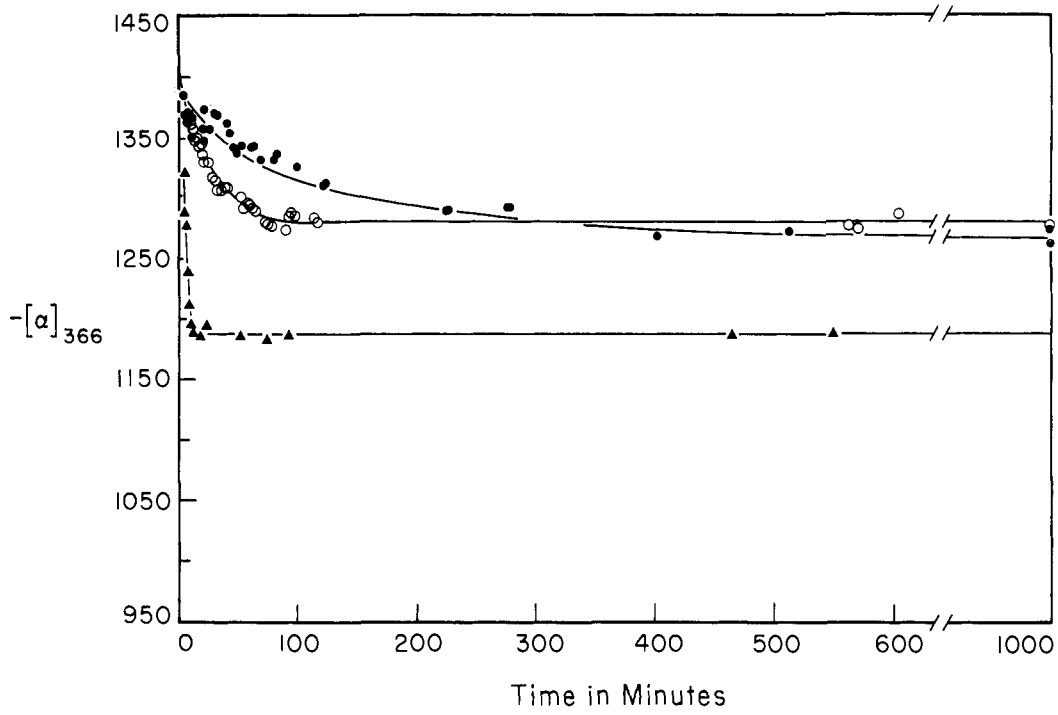

FIGURE 3: Specific optical rotation ($[\alpha]_{366}$) of *Ascaris* collagen as a function of time in the presence of various reducing agents. (●) 0.06 M β-mercaptoethanol, pH 8, 25°. (○) 0.44 M Na_2SO_3–0.004 M PMB, pH 10, 5°. (▲) 0.44 M Na_2SO_3–0.004 M PMB, pH 10, 25°.

a maximum after about 40 min and declining to a negligible value after 400 min. No further changes were detected after 1000 min.

Sedimentation velocity experiments on solutions of the native collagen (0.15%) in 5 M guanidine·HCl revealed a single hypersharp boundary with sedimentation coefficient, $s_{20,w}$ = 7.7 S. After 120 min in the presence of the reducing agent this peak was transformed into a single, slower sedimenting boundary with $s_{20,w}$ = 2.8 S. Thus from the combined sedimentation and viscosity results it is clear that the cross-linked *Ascaris* collagen has undergone a marked decrease in molecular weight in the presence of the reducing agent. (The molecular weight following reduction was estimated at 80,000 from the Scheraga–Mandelkern equation (1953) assuming $\beta = 2.5 \times 10^6$.)

VISCOSITY AND SEDIMENTATION CHANGES IN THE ABSENCE OF DENATURING AGENTS. The physical properties of *Ascaris* collagen are also markedly altered under reducing conditions in the absence of denaturing agents. Figure 2 summarizes the results of an experiment comparable to that presented in Figure 1 where an equivalent volume of β-mercaptoethanol was added to 2 ml of an *Ascaris* collagen solution (2.1 mg/ml) in 0.12 M NaCl–0.2 M Tris·HCl (pH 8.0) at 25°. Addition of the reducing agent was followed by a precipitous decline in viscosity (half-time = 3 min) from an initial value of 32 dl/g to a final value in the range of 1.5 dl/g. Serial dilution of the solution (after 1600 min at 25°) in the viscometer gave an intrinsic viscosity, $[\eta]$ = 1.0 dl/g, and sedimentation *vs.* concentration studies on this material, which sedimented as a single boundary in the ultracentrifuge, showed an $s^0_{20,w}$ = 2.8 S. An estimate of the molecular weight using these parameters gave values in the range 90,000–113,000 (depending on the β factor assumed).

Viscosity changes produced by a number of reducing agents under a variety of temperature, pH, and ionic conditions have been investigated. Results are summarized in Table I and the time dependence of viscosity changes of a few of these reactions are presented graphically in Figure 2 where a wide variation in the half-life of the reaction as well as in the final value of reduced viscosity can be seen. From inspection of Table I it appears that an approximately 1000-fold increase in the rate of the reaction is observed over the pH range 4.6–8.0 in the presence of β-mercaptoethanol. This striking increase in reaction velocity has been observed in a number of disulfide-interchange reactions in this pH range and is consistent with the requirements of an ionized sulfhydryl reagent for nucleophilic attack on protein disulfide linkages (see Cecil, 1963; Cecil and McPhee, 1959). Supporting evidence that disulfide linkages are the fundamental cross bridges under attack in these reactions is provided by the experiments utilizing sodium sulfite.

Although the hydrodynamic properties of *Ascaris* collagen in dilute salt systems undergo marked alterations in the presence of reducing agents, much smaller

TABLE I: Effect of Disulfide Reagents on the Viscosity of *Ascaris* Collagen.

Reducing Agent (M)	Solvent (M)	Temp °(C)	pH	Protein Concn (mg/ml)	Half-Life[a] Reaction (min)	Final η_{sp}/c (min)
β-Mercaptoethanol (0.06)	NaCl (0.12)–Tris (0.2)	25	8.0	2.1	3	1.4 dl/g
(0.06)	NaCl (0.12)–NaAc (0.05)	25	4.55	1.2	4,500	7.1[b] (24,000)
(0.06)	NaCl (0.10)–glycine (0.10)	4.5	8.0	1.2	55	2.7[b] (3,100)
(0.60)	NaCl (1.11)–glycine (0.20)	25	10.0	0.5	≪2	0.9 (3)
β-Mercaptoethylamine (0.05)	NaCl (0.18)–Tris (0.10)	25	8.0	0.8	5	3.0 (300)
Dithiothreitol (0.05)	NaCl (0.18)–Tris (0.10)	25	8.0	0.74	1	1.2 (22)
Sodium sulfite (0.18)	NaCl (0.11)–glycine (0.18)	25	9.0	1.1	800	2.8 (14,000)
Sodium sulfite (0.18) plus PMB (.0018)	NaCl (0.16)–Tris (0.18)	25	8.0	0.74	3	1.1 (35)

[a] Half-life from original viscosity (30–40 dl/g) to 1.1 dl/g. [b] Viscosity still falling.

TABLE II: Molecular Weight of *Ascaris* Collagen Following Disulfide Cleavage under Nondenaturing Conditions.[a]

Reducing Agent (M)	pH	M_w Top	M_w Midpoint	M_w Base	Upward Curvature (log c *vs.* r^2)
β-ME (0.12)	8.4		109,000		Moderate
β-ME (0.12)	8.4	113,000		127,000	Moderate
DTT (0.05)[b]	8.4		126,000		Slight (marked at bottom)
DTT (0.04)[c]	8.4	58,000	84,000	119,000	Marked
DTT (0.04)[d]	8.4	99,000	126,000	190,000	Marked
Na_2SO_3 (0.18)[e] + PMB (0.0018)	8.4		103,000		Slight
Na_2SO_3 (0.44)[e] + PMB (0.0044)	10.0		89,000		None
Na_2SO_3 (0.44)[e] + PMB (0.0044)	10.0	76,000		100,000	Moderate

[a] Solvent was 0.20 M NaCl–0.05 M Tris·HCl or glycine. Rotor velocities of 21,730–24,630 rpm were employed. β-ME, β-mercaptoethanol; DDT, dithiothreitol. [b] Diluted to 0.01 M before ultracentrifugal analysis. [c] Carboxymethylated with iodo-3-acetic acid, acidified, and dialyzed *vs.* 0.18 M NaCl–0.05 M Na-acetate (pH 4.8). [d] Acidified with 3% acetic acid and dialyzed *vs.* 0.18 M NaCl–0.05 M Na-acetate (pH 4.8). [e] Corrected for contribution of protein-bound PMB and sulfite to both protein mass and partial specific volume.

changes are observed in the conformational pattern of the constituent polypeptide chains as judged by optical rotation. Time-dependent changes in the specific optical rotation, $[\alpha]_{366}$, of *Ascaris* collagen in the presence of β-mercaptoethanol and SO_3^{2-}–PMB are shown in Figure 3. The specific rotation decreases to a terminal value which depends on the temperature and reducing conditions, but the total change in rotation ($\Delta[\alpha]_{366}$) is only 15–25% of that observed when the native *Ascaris* collagen undergoes thermal denaturation (Josse and Harrington, 1964). Thus it would appear that a major fraction of the collagen-fold conformation is retained on cleaving the disulfide linkages. The 100° difference in the thermal specific rotation ($[\alpha]^t_{366}$) of the SO_3^{2-}–PMB system at 5 and 25° appears to be due to the tempera-

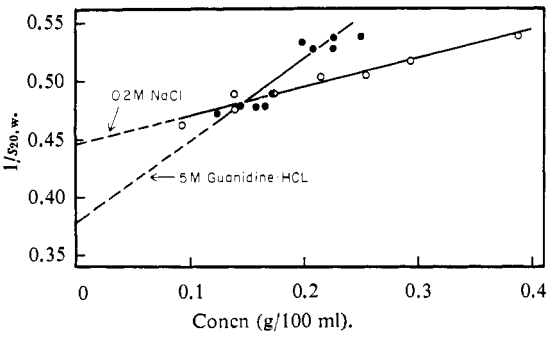

FIGURE 4: Reciprocal of the sedimentation coefficient *vs.* concentration for RCM *Ascaris* in (○) 0.2 M NaCl and (●) 5 M guanidine·HCl at 25°.

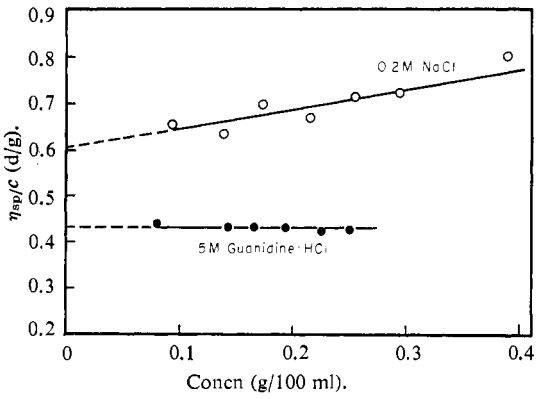

FIGURE 5: Reduced viscosity (η_{sp}/c) *vs.* concentration for RCM *Ascaris* in (○) 0.2 M NaCl and (●) 5 M guanidine·HCl at 25°.

ture dependence of the collagen-fold conformation retained in the reduced product. In this connection it has been observed that the thermal denaturation profile of the isolated product obtained following disulfide reduction (RCM *Ascaris*, see McBride and Harrington (Figure 1), 1967) shows a decrease of about 100° in $[\alpha]_{366}$ over the same temperature span.

Molecular Weight of Ascaris Collagen following Disulfide Cleavage. Solutions of *Ascaris* collagen were examined in high-speed equilibrium sedimentation experiments after treatment with a number of the reagents described in Table I. The reaction was allowed to proceed to completion as determined by viscosity, and the solution diluted to an appropriate concentration with the reducing solvent and dialyzed overnight (25°) against this solvent. Molecular weights on the resulting solutions are present in Table II. Analysis of the mass distribution through the liquid column in these short-column experiments indicated that in general the log c *vs.* r^2 plots were nonlinear and showed varying amounts of upward curvature characteristic of particle heterogeneity. Under the most effective disulfide cleavage conditions (low η_{sp}/c) the weight-average molecular weight (M_w) was significantly less than 100,000 at the midpoint of the liquid column. Those reagents which were less effective in disulfide cleavage, as judged by the terminal viscosity, gave collagen systems with M_w in the range 100,000–125,000.

Physical Properties of Reduced-Carboxymethylated Ascaris Collagen. SEDIMENTATION AND VISCOSITY STUDIES. In order to completely reduce the native collagen and to prevent a dynamic association–dissociation of the liberated polypeptide chains through mercaptide–disulfide interchange, the *Ascaris* collagen was reduced in 5 M guanidine·HCl and the free SH groups were alkylated with iodoacetic acid (Materials and Methods). The resulting solution was dialyzed at 5° against 0.2 M NaCl (5–7 days) and at this time exhibited a specific levorotation of $[\alpha]^5_{366}$ −1050 to −1100°. (Solutions held at 5° for several weeks show an $[\alpha]_{366}$ −1150 to −1200°.) Velocity sedimentation studies demonstrated that the reduced alkylated *Ascaris* (RCM *Ascaris*) migrates as a single, relatively symmetrical boundary at all concentrations examined over the range 0.09–0.4%. A plot of the reciprocal of the sedimentation coefficient *vs.* concentration is shown in Figure 4, yielding an infinite dilution value $s^0_{20,w}$ = 2.24 S. Sedimentation studies were also carried out in 5 M guanidine·HCl in an attempt to further dissociate the polypeptide chain(s) in this denaturing agent. These experiments utilized a single-sector synthetic boundary cell. Sedimentation patterns under these conditions showed a single boundary and no low molecular weight material was detected at the solvent–solution interface. The infinite dilution sedimentation coefficient was $s^0_{20,w}$ = 2.65 S (Figure 4). Intrinsic viscosities of RCM *Ascaris* in 0.2 M NaCl and 5 M guanidine·HCl were determined as outlined in Materials and Methods resulting in values of $[\eta]$ = 0.61 (25°) and 0.43 dl/g, respectively (Figure 5).

SEDIMENTATION EQUILIBRIUM STUDIES OF RCM *Ascaris*. Although the velocity sedimentation patterns of RCM *Ascaris* indicate that this material sediments as a single symmetrical peak, further evidence for particle homogeneity was sought from gel filtration studies utilizing G-200 Sephadex (0.2 M sodium phosphate, pH 6.2). Over 95% of the mass was eluted as a single symmetrical peak in these experiments (see Figure 6). In contrast to native *Ascaris* which eluted at the void volume of these columns, a significant retention of RCM *Ascaris* is seen. It is also of interest that denatured ichthyocol collagen eluted virtually at the void volume of the same column. Since the molecular weight of the α chains of ichthyocol has been established at close to 100,000 (Boedtker and Doty (1956); von Hippel and Wong (1963)) this result points to a lower molecular weight for RCM *Ascaris*.

In view of the apparent absence of gross heterogeneity with respect to size of RCM *Ascaris* in velocity sedimentation and gel filtration experiments, a detailed investigation of the molecular weight of this material was undertaken. The results of both low-speed and high-speed short-column equilibrium sedimentation studies at various temperatures are summarized in

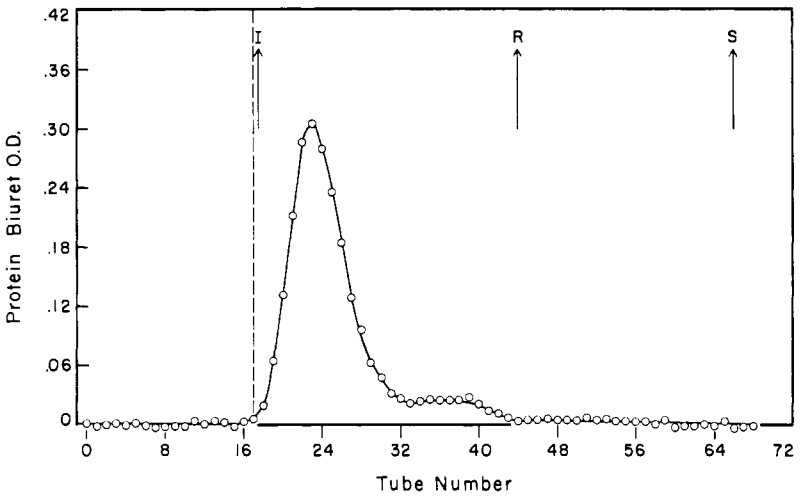

FIGURE 6: Elution profile of RCM *Ascaris* gelatine from a G-200 Sephadex column. The solvent was 0.2 M sodium phosphate (pH 6.2). RCM *Ascaris* solution (0.25%) was heated to 50° for 1 hr then applied to column. The temperature was 25°. I, R, and S indicate the elution position of icthyocol gelatin (50°, 1 hr), RCM ribonuclease, and sodium chloride, respectively. (The vertical dashed line indicates the column void volume; Blue Dextran 2000 elution.)

Table III. Log c *vs.* r^2 plots invariably showed some upward curvature in the low-speed runs but were quite linear in all studies (with one exception) using the high-speed equilibrium technique. A plot of the reciprocal of molecular weight *vs.* concentration obtained from analysis of low-speed equilibrium runs is presented in Figure 7. The molecular weight shows a small but significant concentration dependence and a value of M_w = 62,000 was derived from extrapolation to infinite dilution. A similar plot of the z-average molecular weight, $\bar{M}_z$, yielded an infinite dilution value, $\bar{M}_z$ = 101,000. The observed difference between z- and weight-average molecular weights probably results from a small amount of high molecular weight contaminant since M_w at the midpoint of the column (62,000) was virtually identical with that estimated over the whole column (63,000). Molecular weights derived from high-speed equilibrium experiments gave closely similar values.

It is of special interest that the molecular weight of RCM *Ascaris* is independent of the presence or absence of the collagen-fold conformation. Solutions of RCM *Ascaris* which have been maintained at 5° for several weeks show an $[\alpha]_{366}$ −1200°, *i.e.*, about 75% of the collagen-fold conformation observed in the native *Ascaris* collagen. When the solution is held at 35°, following thermal denaturation at 50° (10 min), $[\alpha]_{366}$ remains essentially invariant at the value characteristic of the random chain conformation ($[\alpha]_{366}$ −660°). Yet both of these systems yield the same molecular

TABLE III: Molecular Weight of RCM *Ascaris* Collagen.[a]

Method	Temp (°C)	Rotor Velocity (rpm)	M_w
Sedimentation equilibrium			
Low speed	25	11,272	62,000 (extrapolated)
High speed	25	24,630	62,000
High speed	25	24,630	62,000
High speed	25	17,580	63,000
High speed	22	24,630	59,000
		29,500	59,000
High speed	5	24,630	63,000
High speed	35[b]	24,630	63,000
High speed	35[b]	24,630	53,000
$s^0_{20,w}$ and $[\eta]$[c]	25	59,780	53,000
$s^0_{20,w}$ and $[\eta]$[c]	25	59,780	72,000[d]

[a] Solvent was 0.20 M NaCl. [b] Heated (50°, 10 min) before centrifugation. [c] Calculated according to the Scheraga–Mandelkern (1953) equation. [d] Solvent was 5 M guanidine·HCl.

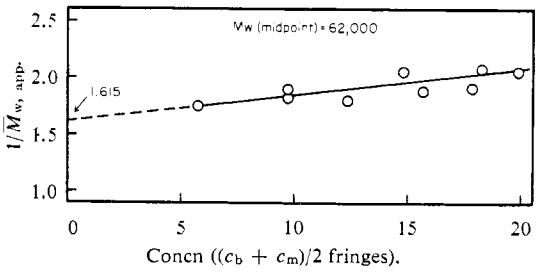

FIGURE 7: Reciprocal of apparent weight-average molecular weight ($\bar{M}_w$) *vs.* concentration in fringes of RCM *Ascaris* in 0.2 M NaCl at 25°.

TABLE IV: Amino Acid Compositions of Native and Reduced Alkylated *Ascaris* Collagens (residues/1000 total residues).[a]

	Native	RCM Soluble[b]	RCM Gel A	RCM Gel B	RCM Gel B[c] Peak 1	RCM Gel B[c] Peak 2
Lys	45	45	45	37	36	43
His	8.4	7.2	8.9	9.7	12.5	18.5
NH_3	49	34	54	46	86	105
Arg	29	29	33	40	26	52
SCMC	0	29.5	28	36.5	36	30.5
Hyp	16	(16)	(16)	(16)	?	?
Asp	69	61	65	65	61	72
Thr	19	13	19	15	15	13
Ser	19	15	22	16	21	16
Glu	67	59	70	68	53	81
Pro	296	319	292	308	328	289
Gly	274	283	257	285	291	294
Ala	72	71	69	63	72	46
$^1/_2$-Cys	27	0	0	0	0	0
Val	18	14	21	15	16	13
Met	5.2	3.7	7.8	6.2	0.8	0.8
Ile	10	8.9	13	12	8.9	13
Leu	19	17	20	16	16.5	13.5
Tyr	3.7	0.9	4.8	0.8	0.8	0.6
Phe	9.9	6.2	8.3	7.2	7.6	4.7

[a] Carbohydrate content of native and RCM (gel B) was 0.24 and 0.17%, respectively, determined by the anthrone method (Loewus, 1952). [b] RCM soluble, RCM (gel A), and RCM (gel B) were prepared by reduction and alkylation of neutral salt-soluble gel A and gel B *Ascaris* preparations, respectively (Materials and Methods). [c] RCM gel B peaks 1 and 2 (see text).

weight. Independent evidence of the invariance of molecular weight during the helix–coil transition in RCM *Ascaris* is also seen in the molecular weight calculated from the sedimentation and viscosity parameters presented earlier. Estimation of the molecular weight of the chain under nondenaturing (0.2 M NaCl) and denaturing (5 M guanidine·HCl) conditions derived from the Scheraga–Mandelkern (1953) equation give molecular weights which are in reasonable agreement with those derived from the equilibrium studies (see Table III).

Chemical Evidence for Disulfide Cross-Linkages. Independent evidence for the presence of disulfide linkages in native *Ascaris* collagen is presented in Table IV. The possible presence of free SH groups was examined by treating a solution of the collagen with iodoacetic acid (300-fold molar excess) in the presence of the denaturing agent 5 M guanidine·HCl. No significant differences between the amino acid composition of this preparation (column 1, Table IV) and that of the native salt-soluble collagens were found. However, when solutions of *Ascaris* collagen in 5 M guanidine·HCl are alkylated following treatment with the reducing agent β-mercaptoethanol, all of the half-cystine residues are quantitatively transformed into SCMC (column 2). The amino acid compositions of *Ascaris* collagen before and after reduction are generally in good agreement with the previously reported composition (Josse and Harrington, 1964) except for the absolute number of half-cystine residues which are significantly higher in the present study.[2] The reason for this discrepancy is not clear at the moment, but independent evidence derived from titration of the SH groups after reduction (Table V) is in agreement with the number of SCMC residues. Moreover, analysis of the native collagen under denaturing conditions (4 or 5 M guanidine·HCl) showed the absence of free SH groups as measured by both DTNB and PMB titration (Table V).

Resolution and Composition of the Polypeptide Chains of Ascaris Collagen. Although it is now well established that the mammalian tropocollagens are composed of three polypeptide chains of similar if not identical molecular weight, the three chains are not identical

[2] Small differences are observed in the tyrosine content before and after alkylation. These are likely to be the result of the formation of iodotyrosine in the presence of the alkylating reagent. Although the total lysine plus arginine content is reproducible throughout the various analyses, a significant and at present unexplained variation in the absolute amount of each of these residues was found.

TABLE V: Sulfhydryl Content of *Ascaris* Collagen in Residues/1000 Total Residues.

	DTNB Assay	PMB Titration	Amino Acid Analysis	
			½-Cys	SCMC
Native	0[a]	—	25–28	0
Native (4 M guanidine·HCl)	0[a]	0[a]	—	—
Reduced gel B	33.9	—	0[b]	36.1[a]
Reduced gel B (nondenaturing)	27.8	—	3.2[b]	33.1[b]
RCM soluble	—	—	0	29.5
RCM gel A	—	—	0	28

[a] As few as 0.5 residue/1000 would have been detectable. [b] Carboxymethylated prior to amino acid analysis.

in primary structure. Present evidence indicates that at least one of the polypeptide chains differs in composition from the other two and in the case of cod skin collagen all three chains have differing primary structures (Piez, 1964, 1965). Ion-exchange column chromatography of RCM *Ascaris* also demonstrates a nonidentity in the chemical structure of the polypeptide chains of *Ascaris* collagen. Figure 8 presents elution profiles obtained on chromatography of RCM *Ascaris* on CM-50 Sephadex. A sharp peak accounting for about 50–55% of the mass of material applied to the column eluted under the ionic conditions of the starting buffer (0.02 M sodium acetate, pH 4.8). This peak showed no significant retardation on the column at lower buffer ionic strengths. In the presence of a salt gradient, 40–50% of the total amount of protein applied to the column was eluted as a second peak at an ionic strength in the range 0.05–0.10 M. Essentially identical results were obtained using CM-cellulose columns.

Amino acid compositions of each fraction isolated from the CM-50 Sephadex columns are given in Table IV. The composition of peak 2 (tubes 82–92) shows a much higher content of all of the basic amino acids (particularly arginine) than does peak 1, consistent with its behavior on cationic-exchange columns. This fraction also contains a higher concentration of the acidic amino acids than does peak 1. Although both peaks 1 and 2 have the same glycine content within experimental error, peak 1 contains a significantly higher proline content and a higher content of half-cystine residues.

Differences in the content of polar amino acids in the basic polypeptide subunits of RCM *Ascaris* were also evident from gel electrophoresis studies. Two major bands of approximately equivalent stain density were observed at pH 4.3 (Methods). At pH 2.5 the more rapidly migrating band was split into two discrete bands, while the slower migrating band remained unaltered in intensity.

Material comprising each peak of Figure 8 was pooled and examined in short-column, high-speed sedimentation equilibrium experiments after dialysis against 0.2 M NaCl. The calculated molecular weights were M_w = 62,900 (peak 1) and 62,600 (peak 2).

Reduction of Matrix Collagen. The reduced carboxymethylated product, whose physical properties are reported in the previous sections, was obtained from the neutral salt-soluble collagen fraction of the *Ascaris* cuticle. It is known from the studies of Watson and Silvester (1959) that the amino acid composition of the whole cuticle is closely similar to that of the neutral salt-soluble fraction, suggesting, as in the case of collageneous tissues from most vertebrate and invertebrate species, that the major fraction of the collagen is disposed in an insoluble, cross-linked matrix. The following experiment demonstrates that cleavage of the disulfide cross-links liberates virtually all of the cuticle collagen in a form indistinguishable from that obtained on reduction of the neutral salt-soluble fraction. Approximately 6 ml of the thick, opaque gel which formed on centrifugation of the whole cuticle homogenate (step A, see Materials and Methods) was suspended in 100 ml of 5 M guanidine·HCl–0.4 M Tris·HCl buffer (pH 8.6). The bulk of the gel was insoluble even on prolonged stirring. A small amount of β-mercaptoethanol (2 ml) was added to this suspension whereupon the gel quickly dissolved leaving a faintly turbid solution. After 16-hr incubation (25°), iodoacetic acid was added to carboxymethylate the free SH groups (Materials and Methods). This solution was dialyzed *vs.* 0.2 M NaCl and, following centrifugation (23,000*g*, 35 min), a small dark pellet and a clear viscous solution were obtained which contained approximately 200 mg of protein. Elution patterns of this material from G-200 Sephadex columns were identical with the RCM *Ascaris* prepared from the neutral salt-soluble fraction.[3] Sedimentation velocity experiments showed a single sedimenting peak with $s_{20,w}$ = 2.20 S (concentration 0.14%) and viscosity measurements gave η_{sp}/c = 0.58 dl/g (25°). The molecular weight of the material was 62,900 by high-speed sedimentation equilibrium analysis and a solution maintained for several months at 5° showed a specific levorotation of $[\alpha]^5_{366}$ −1200°. Similarly, the gel obtained as a residue in the final step (step B) of preparation of the neutral salt-soluble fraction was readily soluble in the presence of β-mercaptoethanol and the product of reduction (in 5 M guanidine·HCl) had physical properties comparable to those obtained above (*viz.*, at a concentration of 1.4 mg/ml, $s_{20,w}$ = 2.2 S; η_{sp}/c = 0.82 dl/g; and M_w = 63,000 in 0.2 M

[3] When material was applied to the column prior to centrifugation virtually all of the cuticle protein was eluted as a single peak at the same elution position as that of the centrifuged material.

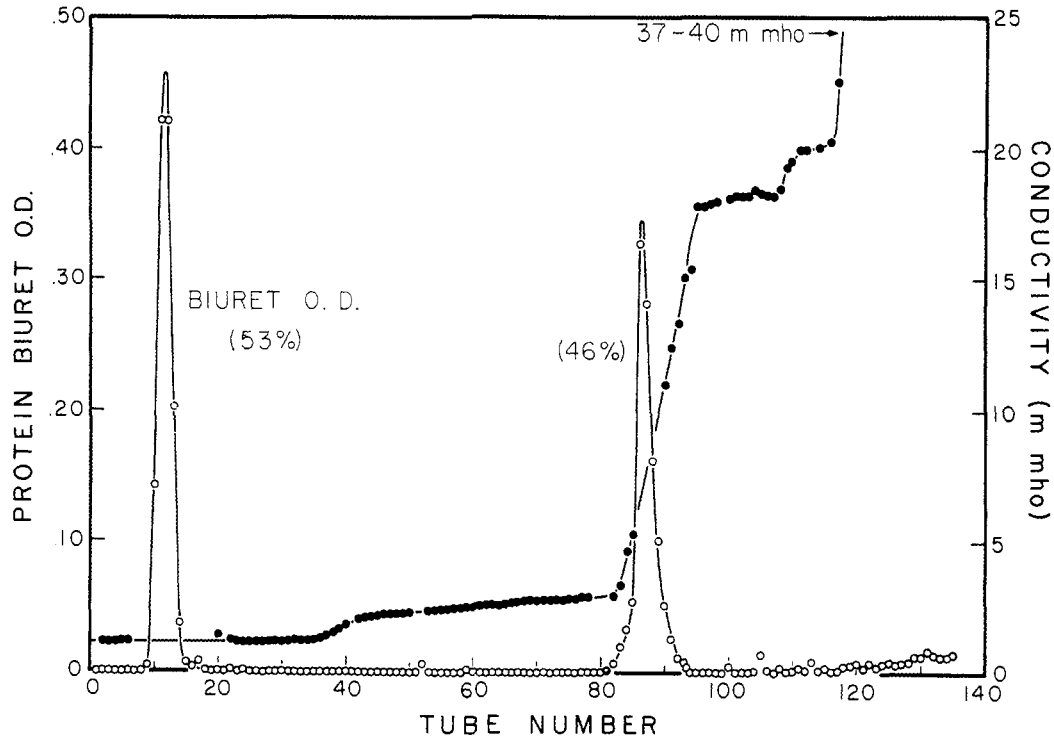

FIGURE 8: Elution profile of RCM *Ascaris* collagen from CM-50 Sephadex. The protein (10 mg) was applied to a 2.5 × 32 cm column and eluted consecutively with 25 ml of 0.02 M Na-acetate (pH 4.8) starting buffer, 250-ml linear gradient 0.02–0.10 M Na-acetate, 150 ml of 0.50 M Na-acetate (pH 4.8), and 190 ml of 0.05 M NaOH–0.50 M NaCl at a flow rate of about 40 ml/hr. Fractions (5 ml) were collected and assayed for (biuret) protein concentration (○) and conductivity (●).

NaCl at 5°). As will be seen from Table IV, the amino acid compositions of the two RCM *Ascaris* materials, prepared under denaturing conditions, are closely similar to that of the neutral salt-soluble fraction.

The whole cuticle homogenate is also soluble under nondenaturing conditions in the presence of reducing agents. Approximately 2 ml of the gel (step A) was suspended in 30 ml of 0.2 M Tris·HCl (pH 8) and β-mercaptoethanol was added (0.06 M). On stirring, the gel quickly dissolved leaving a slightly turbid, viscous solution. This was allowed to stand for 24 hr and then dialyzed against 0.2 M NaCl–0.06 M β-mercaptoethanol (5°). Viscosity and sedimentation studies on the solution gave $[\eta]$ = 1.36 dl/g and $s_{20,w}$ = 2.71 S. The estimated molecular weight using these parameters was 95,000–125,000, that is, in the same range as that calculated for the neutral salt-soluble material in the presence of reducing agents (see Table II).

Reoxidation of Completely Reduced Ascaris Collagen. It was of interest to investigate the possibility of reoxidation of fully reduced *Ascaris* polypeptide chains. An aliquot (6 ml) of the gel obtained in step A (Materials and Methods) was reduced with β-mercaptoethanol in 5 M guanidine·HCl and, following dialysis *vs.* 0.5 M acetic acid (pH 2.5 at 5°), was centrifuged (23,400*g*, 30 min) to remove a small amount of insoluble material. The number of free SH groups on the protein was determined at this point by titration with DTNB giving 32.9/1000 total residues. The reduced viscosity of the solution was 0.73 dl/g (concentration 2.3 mg/ml, 25°). Reoxidation was initiated by dialysis against pH 8.5 buffer (0.15 M NaCl–0.05 M NaH_2PO_4–Na_2HPO_4) at 5°. Aliquots were removed at intervals for viscosity measurement and free SH determination. Results presented in Figure 9 show that although the SH titer decreased about 85% during the 5-day dialysis period, only a relatively small change is apparent in reduced viscosity (from 0.73 to 0.95 dl/g). During the following 5-day period the SH titer dropped to a negligible value (less than 0.3/1000 residues) and the viscosity increased rapidly to about 2 dl/g. Thereafter the reduced viscosity changed very slowly, requiring about 30 days to increase from 2.0 to 2.3 dl/g. High-speed sedimentation equilibrium studies on the reoxidizing system after 7 and 35 days at pH 8.5 revealed significant mass heterogeneity reflected in the upward curvature of the log *c vs.* r^2 plots with molecular weights spanning the range from 72,000 to about 166,000 at the base of the liquid column. A significant thickening of the solution FC-43 interface was observed during the equilibrium sedimentation studies of these solutions suggesting the presence of much higher molecular weight material. Sedimentation velocity experiments revealed the presence of a single-

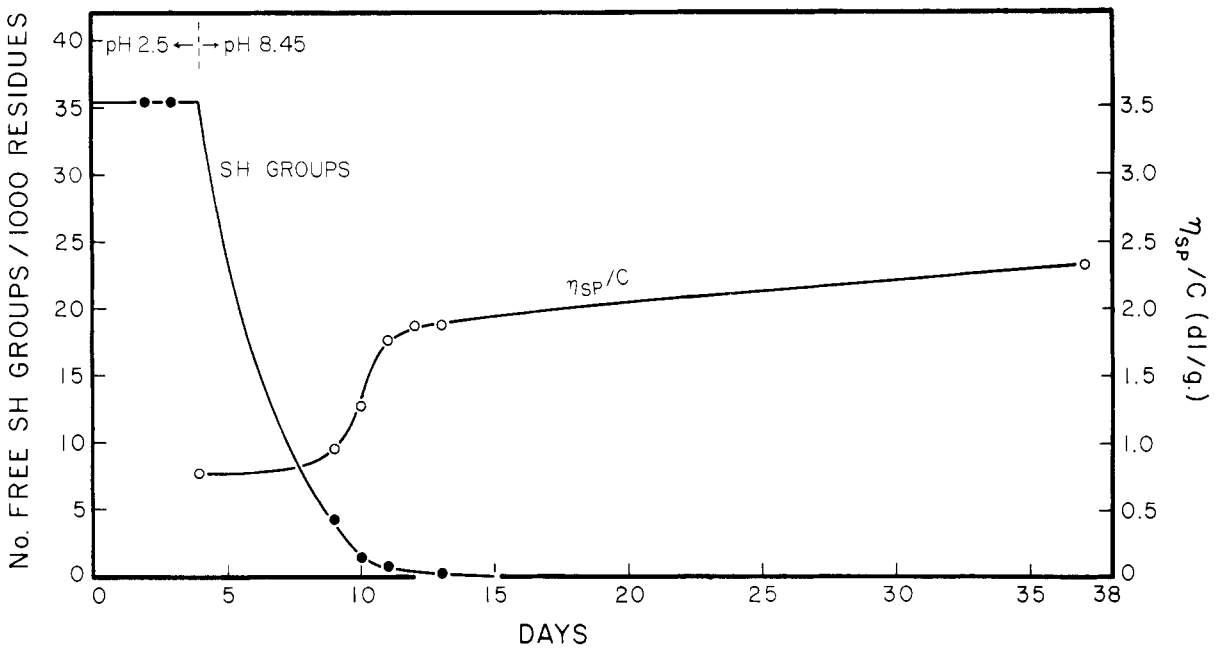

FIGURE 9: Changes in the number of free SH groups (●) and in the reduced viscosity (η_{sp}/C) (indicated as (○)) *vs.* time during spontaneous reoxidation of reduced *Ascaris* collagen at 5°.

TABLE VI: Physical Properties of Native, RCM, and Reoxidized *Ascaris* Collagen.

Solvent (M)	Temp (°C)	$s^0_{20,w}$ (S)	$[\eta]$ (dl/g)	$[\alpha]^t_{366}$	λ_c (mμ)	M_w
			Native			
NaCl (0.2)[a]	5	7.0	12.3	−1,350	201	900,000
Guanidine·HCl (5)[a]	25	9.6	2.9	−710	213	1,100,000
			RCM *Ascaris*			
NaCl (0.2)	5	2.2	0.61	−1,170	202	62,000
	50	~2.5[b]	0.36	−635	213	63,000
Guanidine·HCl (5)	25	2.7	0.43	−640	(224)	73,000
LiBr (8)	25	—	—	−388	214	—
			Reoxidized *Ascaris*			
NaCl (0.2)	5	3.3	2.3	−1,310	199	65,000–165,000 (heterogeneous)

[a] Josse and Harrington (1964). [b] $s^{0.2\%}_{20,w}$ = 2.3 S.

diffuse schlieren boundary in the 35-day system with $s_{20,w}$ = 3.3 S.

The properties of reoxidized *Ascaris* polypeptide chains depend on the conditions used in reduction. *Ascaris* collagen reduced (β-mercaptoethanol) in the *absence* of guanidine·HCl showed 27.8 SH groups/1000 residues and a reduced viscosity, η_{sp}/c = 1.84 dl/g (0.19%), after dialysis against the acetic acid buffer. When this solution was dialyzed against 0.15 M NaCl–0.05 M NaH_2PO_4–Na_2HPO_4 (pH 8.5) for 6 days, the resulting solution was transformed into a clear, thixotropic gel. At this point the SH titer was about 1 SH group/1000 residues. On addition of β-mercaptoethanol (2 μl for 10 ml of a 0.19% protein solution) the gel was rapidly converted into a viscous but free-flowing solution.

Optical Rotatory Dispersion Measurements of Native, RCM, and Reoxidized Ascaris Collagen. Although the hydrodynamic properties of native, RCM, and reoxidized *Ascaris* collagen are markedly different, the fundamental spatial conformation of the polypeptide backbone in these three materials is the same as judged

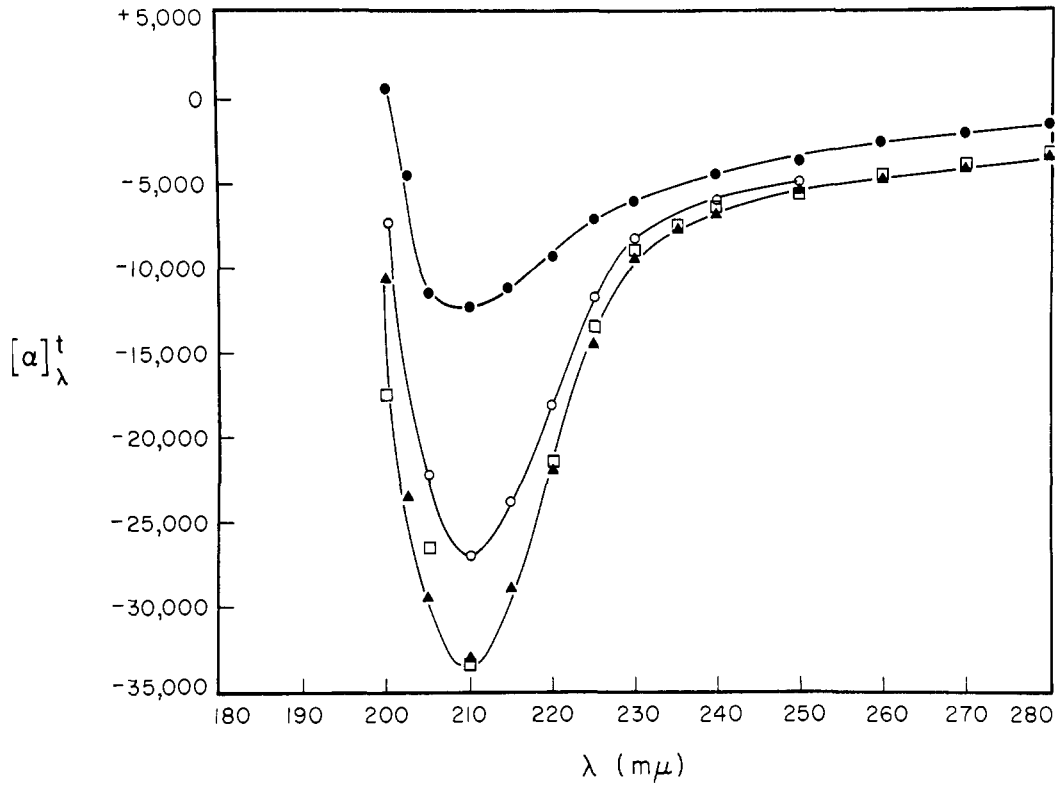

FIGURE 10: Optical rotatory profiles of (▲) native, (□) reoxidized, and (○) RCM *Ascaris* collagen at 5° and (●) denatured RCM *Ascaris* collagen at 40°. Protein concentrations were 0.2–0.3 mg/ml.

by optical rotatory dispersion (Figure 10). At low temperature (5°) all show the characteristic cotton trough of collagen at 208 mμ, and the reoxidized and native forms of *Ascaris* collagen are indistinguishable in their optical rotatory dispersion profiles suggesting that they contain the same fraction of ordered structure. RCM *Ascaris* which has been allowed to renature several weeks at low temperature exhibits a similar optical rotatory dispersion profile, but specific rotation at the cotton trough indicates that only about 70% of the collagen conformation is regained under these conditions. It will also be noted that RCM *Ascaris* which has been heated above the thermal denaturation temperature and then held at a temperature sufficiently high to prevent regeneration of the collagen fold (40°) still exhibits an appreciable cotton trough at 208 mμ. The optical rotatory dispersion profile of the random chain polytripeptide $(Gly\text{-}Pro\text{-}Ala)_n$ shows a similar cotton trough with minimum near 210 mμ (Oriel and Blout, 1966). A summary of the physical properties of native, reoxidized, and RCM *Ascaris* cuticle collagen is presented in Table VI.

Discussion

In view of our finding that disulfide cross bridges play a crucial role in the organization of the polypeptide chains of *Ascaris* cuticle collagen, it is pertinent at this point to recall the evidence that this material is a bona fide member of the collagen group of proteins. The most compelling argument is the wide-angle X-ray diffraction pattern. Faure-Fremiet and Garrault (1944) have reported that the cuticle of *Ascaris* exhibits the typical wide-angle pattern and 2.86-A meridional spacing characteristic of collagen. Moreover, stretched fibers prepared from our salt-soluble material also yield relatively sharp, collagen-type diffraction patterns, although the line intensities and the weak reflections have not been analyzed in detail (G. N. Ramachandran and V. Sasisekharan, private communication). The optical rotatory and infrared properties also show characteristic features generally regarded as strong supporting evidence for the collagen-type structural pattern. These include a specific levorotation, $[\alpha]_D$ −418°; a Drude constant, λ_c 200 mμ (Josse and Harrington, 1964); the presence of a deep, collagen-type cotton trough with minimum at 208 mμ; and a characteristic collagen infrared absorption band centered at 3300 cm^{-1} (Watson and Silvester, 1959). Although the relatively low glycine (270 residues/1000) and hydroxyproline (16 residues/1000) and the remarkably high proline (310 residues/1000) content focus attention on chemical anomalies in the primary structure, the fact is that *Ascaris* does contain high proportions of glycine and the imino residues and is relatively poor in the aromatic amino acids. These are the usual chemical requirements for admission to the collagen class. Most workers have assumed (Harding, 1965; Gallop

and Seifter, 1962; Edsall and Wyman, 1958; Harrington and von Hippel, 1961) that collagen is devoid of cystine cross-linkages and that any cystine found in collagen preparations is probably present as an impurity. In view of the well-known difficulties in the quantitative recovery of cystine in amino acid analyses and the impurity problem it would be understandable for any cystine found to be disregarded. It will be recognized, however, that only a few residues (minimum of 2/3300 total residues) would be required to form a γ-type structure in which three different polypeptide chains are covalently cross-linked. The evidence presented in the present study suggests that changes in the physical properties of collagen in the presence of reducing agents may offer the most sensitive and unambiguous approach to the detection of such cross-linkages.[4]

While all types of collagen fibril give nearly identical wide-angle X-ray diffraction photographs and optical rotatory properties reflecting a close similarity in the spatial conformation of the polypeptide chains, low-angle X-ray patterns show significant variations (Bear, 1952), particularly in the invertebrate collagens, pointing to differences in higher order organization. These variations in small-angle X-ray periodicity are also mirrored in the complex banding pattern observed in electron micrographs of stained collagen. In particular, electron micrographs of *Ascaris* cuticle fibrils are devoid of the characteristic 640-A periodicity commonly observed in the vertebrate collagens (Reed and Rudall, 1948), suggesting that the organization of the triple-helical monomeric units in the collagen matrix may be quite different from that found in the vertebrates. Differences in the organization of the basic collagen units are also emphasized in the present study. As we have seen, the molecular weight of *Ascaris* collagen in the presence of disulfide-cleavage reagents, but in the absence of denaturing conditions, falls to values intermediate between the basic monomeric unit (62,000) and a dimer of this unit, but there is little change in the fundamental chain conformation as measured by optical rotation. This situation stands in contrast to the vertebrate tropocollagens where dissociation of the triple helix into individual chains of molecular weight 100,000 is invariably accompanied by loss of the collagen-fold conformation. Since the reduced viscosity of the *Ascaris* system decreases to values in the range of 0.75–1.1 dl/g under reducing conditions, it is unlikely that the structures released into solution can be single, linear, rigid polypeptide chains folded into the poly-L-proline II type helix or a cross-linked system of two parallel helices. Assuming such particles would behave in solution as rigid ellipsoids, the expected intrinsic viscosities would be of the order of 10 and 6 dl/g, respectively (Simha, 1940). Moreover, neither of these structures would be compatible with the currently

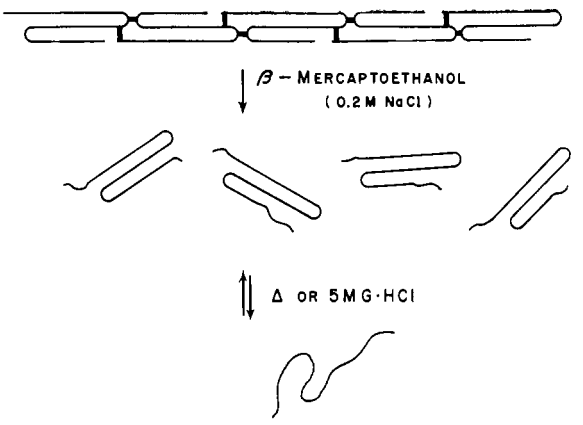

FIGURE 11: A proposed model for the arrangement of polypeptide chains in *Ascaris* cuticle collagen showing the disulfide cross-linkages between subunits in the collagen matrix. Regions of chain overlap within a subunit are thought to be in the triple helical pattern common to the vertebrate collagens. Effect of reducing agents and denaturing agents on the structure is shown in lower portion of the figure.

accepted triple-chain, coiled-coil structure for collagen stabilized by a systematic set of lateral hydrogen bonds. As we have seen, the collagen-type helical conformation of the subunit, RCM *Ascaris*, is unfolded at high temperature or in the presence of guanidine·HCl but reforms at low temperature in dilute salt without any alteration in molecular weight. The final value of specific optical rotation regenerated under these conditions is virtually identical (at a given low temperature) to that obtained on cleaving the disulfide bonds of the native structure. Thus it appears that the individual, monomeric polypeptide units of molecular weight 62,000 can exist in a stable, collagen-type conformational pattern in solution. The question of whether these basic subunits are in reality multichain structures, the individual polypeptide chains being linked through nondisulfide cross bridges, will be considered in the following paper. However, anticipating the results to be presented there, our present evidence favors the view that RCM *Ascaris* exists in solution as a single noncross-linked polypeptide chain. The available evidence seems to indicate that the single-chain forms a stable, hydrogen-bonded, collagen-type, triple-helical structure by folding back upon itself. The intrinsic viscosity of such a structure of molecular weight 62,000 would be about 1.25 dl/g. Thus in the native *Ascaris* structure we suggest that each subunit is a single polypeptide chain of molecular weight 62,000 back-folded to form a three-stranded, hydrogen-bonded super helix, these basic collagen units being held together through disulfide bridges. The collagen subunits would be expected to be released with little change in polypeptide chain conformation on cleaving the disulfide links since the folded single chains are stable structural entities. The possibility that the subunits are released

[4] Blanquet and Lenhoff (1966) have recently reported the presence of a disulfide-linked collagenous protein in the sea anemone nematocysts.

as random chains which subsequently fold into the polyproline II type conformation can be ruled out since in the time required for completion of the disulfide-cleavage reaction at 25° only a trivial amount of the helix can be regenerated.

The significance of the 900,000 molecular weight neutral salt-soluble particles in the matrix structure of *Ascaris* collagen is not clear at the present time. From the earlier hydrodynamic studies these rodlike particles were estimated to be about 4100 A in length and 18 A in diameter (Josse and Harrington, 1964). Assuming that the folded subunit particle of molecular weight 62,000 is a triple helix, its dimensions would be of the order of 500–600 A in length by 13 A in diameter. Thus a subassembly of the appropriate dimensions would permit a linear array of only about seven to eight subunits, suggesting some type of parallel or staggered arrangement. It seems clear that if the subassembly (900,000 mol wt) plays a unique role in the matrix structure, these particles must be covalently linked through disulfide cross bridges since they cannot be dissociated from each other in 5 M guanidine·HCl but are readily disrupted into the subunit particles by reducing agents. Although we have evidence that intersubunit disulfide cross bridges exist in *Ascaris* collagen, the presence of intramolecular (*i.e.*, within a subunit) linkages has not been established. However, examination of both wire and space-filling models shows that a cystine cross-linkage can occur between the individual chains of the triple helix of the common collagen structure without significant perturbation of the spatial geometry.

Acknowledgments

The authors are indebted to Miss Gertrude Karr for assistance with some of the centrifugation experiments.

References

Bear, R. S. (1952), *Advan. Protein Chem. 7*, 69.
Blanquet, R., and Lenhoff, H. M. (1966), *Science 154*, 153.
Boedtker, H., and Doty, P. (1956), *J. Am. Chem. Soc. 78*, 4267.
Boyer, P. D. (1954), *J. Am. Chem. Soc. 76*, 4331.
Cecil, R. (1963), *Proteins 1*, 379, 392.
Cecil, R., and McPhee, J. R. (1959), *Advan. Protein Chem. 14*, 255.
Chinard, F. P. (1952), *J. Biol. Chem. 199*, 91.
Crestfield, A. M., Moore, S., and Stein, W. H. (1963), *J. Biol. Chem. 238*, 622.
Davis, B. J. (1964), *Ann. N. Y. Acad. Sci. 121*, 404.
Edsall, J. T., and Wyman, J. (1958), *Biophys. Chem. 1*, 116.
Ellman, G. L. (1959), *Arch. Biochem. Biophys. 82*, 70.
Faure-Fremiet, E., and Garrault, H. (1944), *Bull. Biol. 78*, 206.
Gallop, P. M., and Seifter, S. (1962), *in* Collagen, Ramanathan, N., Ed., New York, N. Y., Interscience, p 249.
Harding, J. J. (1965), *Advan. Protein Chem. 20*, 109.
Harrington, W. F., and von Hippel, P. H. (1961), *Advan. Protein Chem. 16*, 1.
Josse, J., and Harrington, W. F. (1964), *J. Mol. Biol. 9*, 269.
Kielley, W. W., and Harrington, W. F. (1960), *Biochim. Biophys. Acta 41*, 401.
Lansing, W. D., and Kraemer, E. O. (1935), *J. Am. Chem. Soc. 57*, 1369.
Loewus, F. A. (1952), *Anal. Chem. 24*, 219.
McBride, O. W., and Harrington, W. F. (1965), *J. Biol. Chem. 240*, PC 4545.
McBride, O. W., and Harrington, W. F. (1967), *Biochemistry 6*, 1499 (this issue; following paper).
Oriel, P. J., and Blout, E. R. (1966), *J. Am. Chem. Soc. 88*, 2041.
Ornstein, L. (1964), *Ann. N. Y. Acad. Sci. 121*, 321.
Piez, K. A. (1964), *J. Biol. Chem. 239*, PC 4315.
Piez, K. A. (1965), *Biochemistry 4*, 2590.
Piez, K. A., Weiss, E., and Lewis, M. S. (1960), *J. Biol. Chem. 235*, 1987.
Reed, R., and Rudall, K. M. (1948), *Biochim. Biophys. Acta 2*, 7.
Richards, E. G., and Schachman, H. K. (1959), *J. Phys. Chem. 63*, 1578.
Schachman, H. F. (1963), *Biochemistry 2*, 887.
Scheraga, H. A., and Mandelkern, L. (1953), *J. Am. Chem. Soc. 75*, 179.
Simha, R. (1940), *J. Phys. Chem. 44*, 25.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.
Van Holde, K. E., and Baldwin, R. L. (1958), *J. Phys. Chem. 62*, 734.
von Hippel, P. H., and Wong, K.-Y. (1963), *Biochemistry 2*, 1399.
Watson, M. R., and Silvester, N. R. (1959), *Biochem. J. 71*, 578.
Woessner, J. F., Jr. (1961), *Arch. Biochem. Biophys. 93*, 440.
Yang, J. T., and Doty, P. (1957), *J. Am. Chem. Soc. 79*, 761.
Yphantis, D. A. (1964), *Biochemistry 3*, 297.
Zamenhof, S. (1957), *Methods Enzymol. 3*, 696.